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Acute Toxicity and Efficacy of Current Medical Countermeasures against VM in Guinea Pigs: A Comparison to VX and VR

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14. ABSTRACT
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Abstract

VX and Russian V-agent (VR) are the two most widely known V-agents; however, others also exist. One of these is o-ethyl S-[2-(diethylamino)-ethyl]methylphosphono-thioate, or VM. These studies investigated the lethality of VM in guinea pigs following subcutaneous challenge and determined the effectiveness of current U.S. medical countermeasures atropine sulfate, pralidoxime chloride (2PAM) and diazepam with and without pretreatment with pyridostigmine bromide. The 24 hr median lethal dose (MLD) of VM was determined using a sequential stage approach. The efficacy of medical countermeasures was determined against 3.5 or 5 times the MLD in saline- or PB-pretreated animals. Treatment with atropine + 2PAM + diazepam was administered i.m. 1 min after VM challenge. Survival was assessed at 24 hr. Efficacy studies were conducted also with VX and VR for comparison. The 24 hr MLD of VM was 14.9 ug/kg. Medical countermeasures were equally effective against VM and VX with 90-100% survival against 3.5 MLD VM, and 60-90% survival against 5 MLDs. Medical countermeasures were less effective against VR.

Introduction

Chemical warfare nerve agents are grouped into two general classes: G-agents and V-agents. The G-agents are sarin (GB), soman (GD), cyclosarin (GF) and tabun (GA). VX and Russian V-agent (VR or R-VX) are the most widely known V-agents, but others are known to exist.[1,2] One of the other known V-agents is o-ethyl S-[2-(diethylamino)-ethyl]methylphosphonothioate (VM). The structure of VM, as well as of VX and VR, is shown in Table 1. VM has structural similarities to both VX and VR. VM and VX both have an o-ethyl group attached to the phosphorus, while VR has an o-isobutyl group. On the nitrogen end of the molecule, VM and VR have a diethyl substituent, while VX contains a diisopropyl substituent. Other than its structure and some toxicity data not much is known about VM.[1,2] There are no known data on the effectiveness of medical countermeasures (MC). In this study we determined the s.c. lethality of VM in guinea pigs and evaluated the effectiveness of the current U.S. medical countermeasures, atropine sulfate (Atr), pralidoxime chloride (2PAM), and diazepam (DZ), with and without pyridostigmine bromide (PB) pretreatment against lethal intoxication with VM, VR or VX.

Methods

Animals: Adult male Hartley outbred guinea pigs (250-350 gm) (Charles River, Canada) served as subjects. Animals were maintained in quarantine for 5 days after arrival and prior to use in these experiments. Food and water were available *ad libitum*. Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals. The animal protocol was approved by the Institutional Animal Care and Use Committee.

Chemicals: VM, VX and VR were obtained from the U.S. Army Edgewood Chemical Biological Center (ECBC), Aberdeen Proving Ground, Maryland. Gravimetric concentrations up to 1 mg/ml were prepared in saline and stored in aliquots at -80°C until use. An aliquot was thawed on the day of study and further diluted in saline for injection. The V-agents were injected s.c. between the shoulder blades at a volume of 1 ml/kg. Pyridostigmine bromide USP (PB) was purchased in 5 mg/ml, 2 ml vials and diazepam USP (DZ) in 5 mg/ml, 10 ml multiuse bottles from TW Medical (Lago Vista, TX). PB was further diluted to 1 mg/ml aliquots in sterile water and frozen at -4^{o}C. On each study day an aliquot was thawed and diluted for injection in sterile water. A diazepam aliquot was withdrawn from the bottle and diluted with a vehicle consisting of 40% propylene glycol, 10% ethanol, 1.5% benzyl alcohol, and 48.5% sterile water on the day of study. Crystalline atropine sulfate USP (Atr) and pralidoxime (2PAM) chloride USP were purchased from Spectrum Chemicals (New Brunswick, NJ). Atropine and 2PAM stock solutions were prepared in sterile water and stored in the refrigerator. Atropine and 2PAM were admixed and administered as a single injection on the day of study. PB and diazepam were each administered as separate injections. All therapeutic drugs were injected i.m. in the left and right hind limbs at a volume of 0.5 ml/kg.

Experimental Design:

Median Lethal Dose (MLD) Studies: The toxicity of VM was determined using a stagewise, adaptive dose design.[3,4,5] The experiment was comprised of 3 stages. Stage 1 consisted of 5 VM doses spanning the range of projected nonlethal to lethal doses with 2 animals per dose. Based on the 24 hr lethality results, stage 2 and 3 VM doses were selected to complement the results of the previous stage(s). In stages 2 and 3, 1-3 animals were assigned to each of 4-7 challenge levels of VM. A total of 30 animals were used. After the last stage least squares probit models were fitted to the data using SAS NLIN to calculate the MLD and 95% confidence intervals.

Medical Countermeasure Studies: Animals were pretreated i.m. with PB (0.026 mg/kg) or saline 30 min prior to V-agent challenge. This dose of PB results in 20-30% inhibition of red blood cell (RBC) acetylcholinesterase (AChE) within 30 min (MRICD unpublished data). Animals were challenged s.c. between the shoulder blades with 3.5 or 5 times the MLD of VM, VX or VR. One minute later, each animal was treated i.m. in a hind limb with Atr (0.5 mg/kg) plus 2PAM (25 mg/kg). DZ (0.72 mg/kg) was injected i.m. in the opposite hind limb immediately after Atr and 2PAM. The Atr dose and DZ dose were the human equivalent of 6 mg (3 autoinjectors) and 10 mg (1 autoinjector) respectively, based on body surface area (BSA) formula guidance of the Food and Drug Administration (FDA). The 2PAM dose was the human mg/kg body weight equivalent of 1800 mg (3 autoinjectors). Survival was assessed at 24 hr after agent challenge.

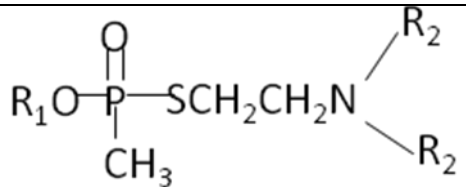
Data Analysis: The survival proportions in the various treatment groups were compared using the Marascuilo multiple proportion test.[6] Statistical significance was defined as $p \leq 0.05$.

Results

MLD Studies. The VM dose lethality curve is shown in Figure 1. The 24 hr MLD was determined to be 14.9 ug/kg, s.c., and the 95% confidence limits were 12.9 ug/kg and 18.9 ug/kg. The slope of the dose lethality curve was 11.4.

Medical Countermeasure Studies. Twenty-four hr survival rate results are shown in Table 2. Treatment against 3.5 or 5 MLDs of VM with self-buddy aid equivalent doses of Atr, 2PAM and DZ with or without PB pretreatment resulted in survival rates of 60-90%. The efficacy of medical countermeasures against VM was similar to the efficacy of the regimens with or without PB pretreatment against VX. The current medical countermeasure treatment regimen was significantly less effective against VR in animals challenged with 3.5 or 5 MLDs without PB pretreatment and against 5 MLDs with PB pretreatment.

Table 1: Structures of V-agents



Name	R_1	R_2
VX	ethyl	isopropyl
VM	ethyl	ethyl
VR	isobutyl	ethyl

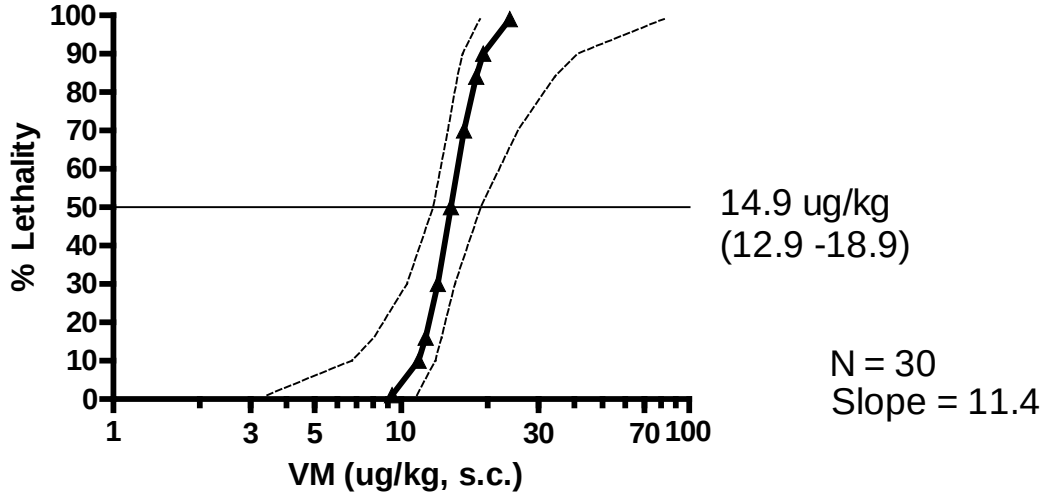


Figure 1: Subcutaneous toxicity of VM in guinea pigs. Graph shows the 24 hr dose lethality curve (solid thick line) and lower and upper 95% confidence limits (dashed lines) calculated by probit analysis. The horizontal thin line intersects the MLD and the 95% confidence limits for the MLD.

Table 2: Efficacy of current medical countermeasures against V-agent intoxication in guinea pigs

V-agent	Pretreatment[1]	**24 hr Survival Rate** following i.m. Treatment with Atr (0.5) + 2PAM (25) + Diazepam (0.72) 1 min after challenge[2]	
		3.5 MLD, s.c.	5 MLD, s.c.
VM	PB	9/10	6/10
	Saline	9/10	8/10
VX	PB	9/10	9/10
	Saline	10/10	9/10
VR	PB	5/10	1/10*
	Saline	2/10*	2/10*

1. PB or saline administered i.m. 30 min prior to V-agent. PB dose was 0.026 mg/kg, which results in 20-30% inhibition of RBC AChE at the time of agent challenge.
2. Drug doses of medical countermeasures are in mg/kg and are the human equivalent of 3 ATNAAs + 1 CANA.
* $p<0.05$ compared with VM and VX

Table 3: Comparative toxicity of V-agents in guinea pigs

V-agent	24 hr LD 50	95% CI	slope	N
VM	14.9	12.9 - 18.9	11.4	30
VX[1]	9.0	8.6 - 9.4	14.8	194
VR[1]	11.3	10.5 – 12.1	19.5	56

Discussion

The MLD of VM determined in this study was higher than what we have previously found for either VX or VR in guinea pigs (Table 3). Compared to the historical values, VM was 1.65-fold less toxic than VX and 1.3-fold less toxic than VR. Based on non-overlapping 95% confidence limits for each of the agents, VM looks like it is significantly less toxic than either VX or VR. However, previous reports on the toxicity of VM have had variable results relative to other V agents. One report found VM to be more toxic than VX when injected parenterally (i.m., s.c. or i.v.) in mice and less toxic in rabbits and guinea pigs.[1] In rats, the i.m. MLDs of the three V-agents ranked in the same order as observed in the present study.[2] By the percutaneous route VM was reported to be more toxic than VX in dogs, mice and rabbits and less toxic than VX in guinea pigs.[1] It would seem that more toxicity studies with VM are needed to definitively characterize its relative toxicity compared to other V-agents. In any case, VM was at least 2-fold more toxic than any of the G-agents in guinea pigs.[7]

VM-intoxicated animals responded to current medical countermeasure treatment similarly to VX-intoxicated animals. High survival rates were observed against both 3.5 and 5 MLD challenges of either agent in animals treated with self-buddy aid, human equivalent doses of the medical countermeasures. While all the components in the treatment regimen act synergistically to promote survival, reactivation of VM- and VX-inhibited AChE by 2PAM at peripheral nerve synapses is probably primarily responsible for the high survival rates observed in these studies. The same treatment regimen was not very effective against VR, which is known to be more resistant to reactivation with 2PAM.[8] While there have been no oxime reactivation studies on VM reported in the literature to our knowledge, the survival results suggest that 2PAM should be a good reactivator of VM-inhibited AChE.

PB-pretreated animals poisoned with VX and treated with atropine plus 2PAM have been previously reported to exhibit significantly lower protective ratios than non-PB-pretreated animals.[9] In the present study, animals pretreated with PB exhibited a slightly but not significantly lower survival rate against 5 MLDs of VM compared to animals not pretreated with PB. A lower survival rate was not observed at 3.5 MLDs VM, and was also not observed in VX-challenged animals at either exposure level. The latter findings are in conflict with the previously mentioned report with VX. The apparent reduction in survival rate may be an artifact of the small sample size (N=10) of the treatment group. Additional studies should be conducted to determine the significance of the apparent decrease in survival of PB-pretreated animals poisoned with 5 MLDs of VM.

The results of this study and previously published findings show that VR intoxication is significantly more difficult to treat than either VX or VM intoxications with MC regimens containing 2PAM as the oxime. As mentioned above, this is probably due to the resistance of VR-inhibited AChE to reactivation by 2PAM. It may be that the presence of the –isobutyl group on the phosphorus atom of VR affects the ability of 2PAM to dephosphonylate and reactivate inhibited AChE. Other oximes, however, such as HI6 and MMB4, have been reported to be much better reactivators of VR-inhibited AChE.[10]

In summary, VM is a highly toxic organophosphorous compound which appears to be easily treatable with current medical countermeasures following s.c. challenge in guinea pigs. Additional studies are needed to further characterize the toxicity of this V-agent by the percutaneous route and the response to medical decontamination. Additional medical countermeasure studies should focus on reactivation of VM-inhibited AChE by oximes, effectiveness following percutaneous exposure and the risk benefit of using PB pretreatment.

References

1. Vachek J, Gersl V, Fusek J, Krs O, Skopec F, and Bajgar J. (1996) Toxicities of o-alkyl s-(2-dialkylaminoethyl) methyl phosphonothiolates (V-compounds). Acta. Medica (Hradec Kralove) 39: 67-71.

2. Bajgar J, Kuca K, Fusek J, Karasova J, Kassa J, Cabal J, Jun D, and Blaha V (2007). Inhibition of blood cholinesterase following intoxication with VX and its derivatives. J. Appl. Toxicol.

3. Feder PI, Hobson DW, Olson CT, Joiner RL, and Mathews MC (1991a). Stagewise adaptive dose allocation for quantal dose-response studies. Neurosci. Biobehav. Rev. 15: 109-114.

4. Feder PI, Olson CT, Hobson DW, Mathews MC, and Joiner RL (1991b). Stagewise, group sequential experimental design for quantal responses: one-sample and two-sample comparisons. Neurosci. Biobehav. Rev. 15: (129-133).

5. Feder PI, Olson CT, Hobson DW, Mathews MC, and Joiner RL (1991). Statistical analysis of dose-response experiments by maximum likelihood analysis and iteratively reweighted least squares regression techniques. Drug Info. J. 25: 323-334.

6. Marascuilo, LA (1966). Large-sample multiple comparison. Psychological Bulletin, 65: 280-290.

7. Shih T-M, Kan RK, and McDonough JH (2005). In vivo cholinesterase inhibitory specificity of organophosphorus nerve agents. Chem-Bio. Interact. 157-158: 293-303.

8. Maxwell DM, Brecht KM, and Koplovitz I (1997) Characterization and treatment of the toxicity of O-isobutyl s-[2-(diethylamino)ethyl]methylphosphonothioate, a structural isomer of VX, in guinea pigs. J. Am. Coll. Toxicol. 15: (Suppl 2) s78-s88.

9. Koplovitz I, Harris LW, Anderson DR, Lennox WJ, and Stewart JR (1992). Reduction by pyridostigmine pretreatment of the efficacy of atropine and 2PAM treatment of sarin and VX poisoning in rodents. Fund. Appl. Toxicol. 18: 102-106.

10. Luo C, Tong M, Chilukuri N, Brecht, K, Maxwell DM, and Saxena A (2007). An in vitro comparative study on the reactivation of nerve agent-inhibited guinea pig and human acetylcholinesterase by oximes. Biochem. 46: 11771-11779.